Lipids (2007) 42:1073–1084
DOI 10.1007/s11745-007-3107-8

REVIEW

A Practical Guide to the Isolation, Analysis and Identification of Conjugated Linoleic Acid

William W. Christie · Gary Dobson · Richard O. Adlof

Received: 12 March 2007 / Accepted: 25 July 2007 / Published online: 21 August 2007

Abstract Natural and synthetic conjugated linoleic acids (CLA) are reputed to have therapeutic properties that are specific to particular geometrical and positional isomers. Analysis of these has presented unique problems that have brought forward distinctive solutions, especially the use of gas chromatography–mass spectrometry and silver-ion high-performance liquid chromatography. In the analysis of CLA present at low levels in tissue samples, it is sometimes necessary to use concentration methods. In this review, the most useful and practical methods for the isolation and analysis of CLA isomers in tissues and in commercial CLA preparations are described.

Introduction

There is increasing interest in geometric and positional isomers of conjugated linoleic acid (CLA) because of their potential therapeutic properties [1–3]. Natural CLA is known to consist of several geometrical isomers of which the most abundant is 9-*cis*,11-*trans*-octadecadienoic acid, formed by biohydrogenation in the rumen of ruminant animals. Commercial CLA is produced by alkaline isomerization of linoleate-rich oils, such as sunflower oil, and tends to contain an equimolar mixture of 9-*cis*,11-*trans*- (9*c*,11*t*-) and 10-*trans*,12-*cis*-octadecadienoic acids (10*t*,12*c*-18:2), together with variable amount of other geometrical and positional isomers. In addition, these isomers can be elongated and desaturated in animal tissues by the enzymes involved in the biosynthesis of longer-chain omega-6 fatty acids to produce conjugated analogues, which may even be responsible for some of the biological activity of CLA [4]. In analysing CLA, it is therefore, important that we be able to separate and quantify these geometrical and positional isomers, avoiding additional isomerization during any derivatization steps. Analytical methodology is especially important now that it is recognized that the various isomers may have very different effects in biological systems. Many of the chapters in recent AOCS monographs [1–3] deal with this topic at length.

In considering the analysis of CLA, it is useful to treat the subject from two practical viewpoints. Commercial CLA is usually supplied as the free acid or ethyl ester with the components of interest being present at high levels, so analysis is relatively straightforward, and is discussed briefly here. In animal tissues, CLA is in esterified form and tends to be present at relatively low levels, so concentration steps, for example by reversed-phase or silver ion chromatography, may be required for characterization and analysis. However, some steps may be common to both aspects of the problem. Capillary gas chromatography (GC) with columns and polar phases of the type used for the analysis of *trans* fatty acids, e.g., CP-Sil 88^{TM}, SP2380^{TM} or BPX-70^{TM} (100 m), form the basis of the preferred approach, with GC–mass spectrometry (MS) as the 4,4-dimethyloxazoline (DMOX) derivatives and/or

A more comprehensive list of references to the analysis of CLA is available online: http://www.lipidlibrary.co.uk/.

W. W. Christie (✉)
MRS Lipid Analysis Unit, c/o SCRI,
Dundee DD2 5DA, Scotland, UK
e-mail: William.Christie@scri.ac.uk

G. Dobson
SCRI, Dundee DD2 5DA, Scotland, UK

R. O. Adlof
USDA, NCAUR, Agricultural Research Service,
Peoria, IL 61604, USA

4-methyl-1,2,4-triazoline-3,5-dione (MTAD) adducts for definitive identification of double bond positions. In addition, silver ion high-performance liquid chromatography (HPLC) has proved very useful for the separation of geometrical and positional isomers both on analytical and preparative scales.

In this review, we have been pragmatic and highly selective in describing those methods that appear to us to be most useful for isolation and analysis of CLA and CLA isomers, as opposed to an exhaustive discussion of the literature. There is inevitably an element of subjectivity in our approach. Most analysts will employ GC for routine analysis of CLA and this may be supplemented with mass spectrometry for definitive identifications. Silver ion high-performance liquid chromatography is an invaluable alternative or complementary procedure. However, CLA levels in tissues of foodstuffs may be too low to be directly amenable to such analyses. Then, pre-concentration methods may have to be applied. Detailed protocols for many of the methods described here are available in earlier reviews [3, 5].

Preparation of Methyl Ester Derivatives of Lipids Containing CLA

The first step before chromatographic analysis of lipids containing CLA is preparation of the methyl ester derivatives. It is well established that acid-catalysed transesterification of lipids can bring about isomerization of *cis*,*trans*- to the *trans*,*trans*-forms with some loss of CLA, so that base-catalysed methods are most suitable for the purpose [6]. Sodium methoxide in anhydrous methanol is the preferred reagent for glycerolipids. Free fatty acids cannot be esterified by this means, and while trimethylsilyl-diazomethane is occasionally recommended, there is ample evidence that it can lead to artefact formation. However, free fatty acids can be methylated by acid-catalysed procedures if the reaction time is kept short. For example, boron trifluoride–methanol or sulfuric acid (1% v/v)–methanol reagents can be employed provided that scrupulous attention is paid to detail [5]; in particular, freshly prepared reagent should be used and the reaction time kept to the minimum. For example, free fatty acids are fully and safely methylated with boron trifluoride–methanol in 10 min at ambient temperature (or with sulfuric acid–methanol (1%) at 50 °C for up to 1 h). Methods of preparing methyl esters have been reviewed exhaustively elsewhere [7].

Gas Chromatographic Analysis

Gas chromatography is by far the most widely used method for the analysis of the total CLA content of samples and for the composition of individual isomers. Coupled to mass spectrometry for identification purposes, it is an extremely useful tool. It is recognized that long (50 or 100 m) polar columns coated with 100% cyanopropyl polysiloxane (e.g., CP-Sil 88^{TM}, SP2380^{TM} and SP 2560^{TM}) give optimum resolution of CLA isomers. Under optimal conditions, methyl esters of CLA isomers elute just after 18:3(n-3) and 20:1 isomers and before 20:2(n-6), although under some conditions 20:1 isomers can interfere with the analysis of CLA. On a 100 m CP-Sil 88^{TM} column, the following conditions have been used successfully for total cheese fatty acid methyl esters (FAMEs); holding the temperature at 75 °C for 2 min, temperature programming at 5 °C/min to 180 °C, holding at 180 °C for 33 min, then at 4 °C/min to 225 °C and finally holding at 225 °C for 44 min [8]. Under these conditions the CLA isomers eluted between 49 and 53 min. The low starting temperature enabled short-chain fatty acids to be separated, but a higher starting temperature (150–175°C) can be used for most other tissue samples. If the total CLA content is all that is required, a column of medium polarity (CP-Wax 58^{TM}) and moderate length (25 m) can be used, the CLA region emerging after 18:3(n-3), and before all C_{20} and C_{21} FAMES.

The distribution of CLA isomers in commercial preparations has been examined by GC of the methyl esters [with identification by GC–MS of the DMOX derivatives (see below)] [9]. Typical commercial CLA mixtures contain mainly *cis*,*trans/trans*,*cis* isomers and less *cis*,*cis* and *trans*,*trans* isomers (Fig. 1). The *trans*,*cis* CLA isomers elute before the *cis*,*cis* and finally the *trans*,*trans* isomers emerge. Usually only one of the *cis*,*trans/trans*,*cis* forms (e.g., 9*c*,11*t*-18:2 but not 9*t*,11*c*-18:2) of each positional isomer occurs. The main isomers are 9*c*11*t*- and 10*t*12*c*-18:2 in roughly equal amounts, and 8*t*10*c*- and 11*c*13*t*-18:2 may be either absent or present to varying degrees. The 8*t*10*c*-18:2 isomer is sometimes totally masked by the 9*c*11*t*-18:2 isomer but can be seen as a definite later-eluting shoulder or even as a split peak when the proportion of the two isomers is similar. The 11*c*13*t*-18:2 isomer emerges next and may not be totally resolved from the 10*t*12*c*-18:2 isomer. Trace amounts of 10*c*,12*t*- and 9*t*,11*c*-18:2 isomers are sometimes apparent, occurring between the 9*c*,11*t*-/8*t*,10*c*- and 11*c*,13*t*-18:2 peaks. Low levels of *cis*,*cis* isomers are reasonably well resolved (elution order 8,10- < 9,11- < 10,12- < 11,13-18:2) occurring just after the 10*t*,12*c*-18:2 peak. Within the *trans*,*trans* isomers the 11,13-18:2 isomer elutes ahead of the other (8,10-, 9,11- and 10,12-18:2) poorly resolved isomers.

Similarly, GC (and GC–MS of DMOX derivatives) has been utilized for characterizing CLA in a variety of natural samples. The materials may be analysed without pretreatment or after concentration of total CLA by RP–HPLC (see below) or, for the most detailed examination as has

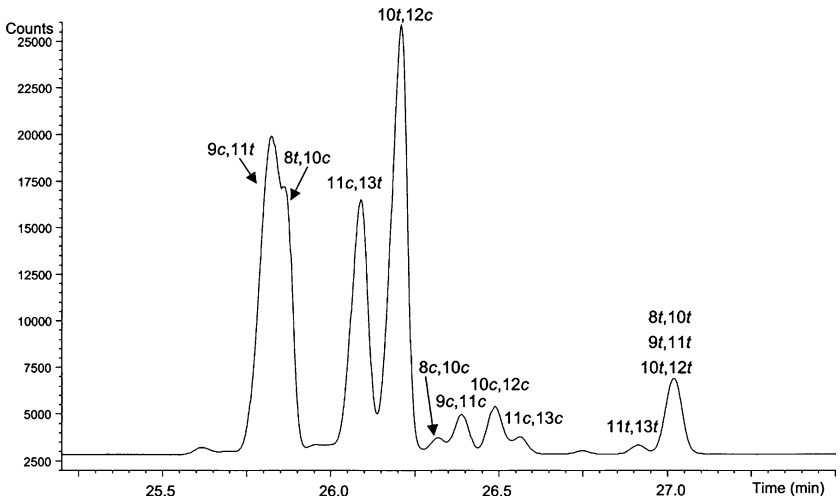


Fig. 1 Partial gas chromatogram of a commercial CLA methyl ester mixture, separated on a CP-Sil 88 capillary column (100 m × 0.25 mm id × 0.2 μm film thickness; Chrompack UK Ltd, London, UK) with flame-ionization detection. The oven temperature was held at 160 °C for 3 min, then was programmed at 2 °C/min to 220 °C and held at 220 °C for 20 min. Hydrogen was the carrier gas at a constant flow rate of 1 ml/min and a split ratio of 50:1 was used

been applied to cheese, after fractionation of the CLA by silver-ion HPLC (discussed below). In the latter case it is interesting that a short non-polar (30 m HP5MS) column can be used for CLA analysis. It would appear that optimum resolution by GC may not be essential if pre-fractionation by silver ion HPLC is carried out.

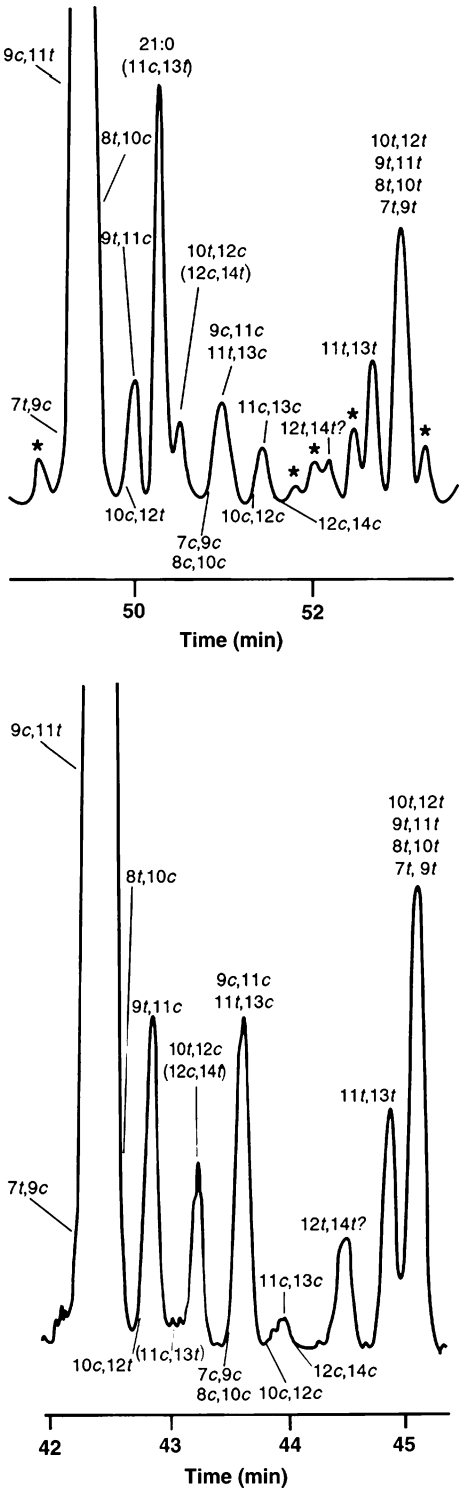
Natural samples for CLA analysis often contain more isomers than commercial CLA and the proportion of isomers within each sample type is different, affecting peak resolution. Invariably the 9*c*,11*t*-18:2 isomer is the major peak representing 80–90% of total CLA isomers. The isomer distribution in cheese contains all the isomers found in other foods (Fig. 2) [10, 11]. The *cis*,*trans/trans*,*cis*, *cis*,*cis* and *trans*,*trans* groups all contain positional isomers ranging from 7,9- to 12,14-18:2. Within the *cis*,*trans/trans*,*cis* isomers, in contrast to most commercial CLA, all possible geometrical isomers have been detected with the exception of 8*c*,10*t*- and 12*t*,14*c*-18:2. Retention times increase with increasing distance of the *cis* double bond from the ester moiety, and for isomers that have the same *cis* double bond, with the exception of the 10*t*,12*c*-/12*c*,14*t*-18:2 pair, the isomer with the *trans* double bond nearest the ester moiety elutes first. In cheese, none of the *cis*,*trans/trans*,*cis* isomers elute as pure peaks; 7*t*,9*c*-, 8*t*,10*c*- and 9*c*,11*t*-18:2 overlap, as do 9*t*,11*c*- and 10*c*12*t*-18:2, and 10*t*12*c*- and 12*c*14*t*-18:2, and 11*t*13*c*-18:2 overlaps with the first *cis*,*cis* peak (Fig. 2). Within the *cis*,*cis* isomers, presumably due to the wide range in the relative amounts of the different positional isomers, only two peaks are apparent, the first comprising the 7,9- to 9,11-18:2 isomers, the second the 10,12- to 12,14-18:2 isomers. The 12*t*,14*t*-18:2 isomer elutes first followed by the 11*t*,13*t*-18:2 isomer and then the major *trans*,*trans* peak, comprising 7*t*,9*t*-10*t*,12*t*-18:2.

A CLA standard mixture for is essential for identifying CLA isomers in natural samples by GC. A commercial CLA mixture is useful but it does not contain all the isomers found in natural samples. Other standard mixtures can be prepared. Preparations of mixtures containing all 16 possible isomers ranging from 8,10- to 11,13-18:2, obtained by isomerization of commercial CLA with either iodine or *p*-toluenesulfinic acid as catalyst [12], or with additional 7*t*,9*c*- and 12*c*,14*t*-18:2 isomers using a procedure involving sigmatropic rearrangements and selenium-catalysed geometrical isomerization [13], are useful as standards to assist identifications. All 32 possible isomers from 6,8- to 13,15-18:2 have been synthesised and the relative retention times determined on a 100 m CP-Sil 88™ [14] (Table 1).

Several peaks (e.g., 21:0) that are not CLA isomers may occur in the CLA region of the gas chromatogram of natural CLA samples, and in samples extracted from tissue derived from animals fed commercial CLA. The 21:0 is often at similar concentrations to the minor CLA isomers and can elute anywhere between 11*c*,13*t*- (as in Fig. 2) and 10*c*,12*c*-18:2 depending on the GC conditions [15]. Care has to be taken to avoid misidentifications, and use of columns differing in polarity can remove some dubiety. Mass spectrometry can eliminate all doubts [8].

Silver Ion High-Performance Liquid Chromatography

Silver-ion high-performance liquid chromatography (Ag-HPLC) complements gas chromatographic analysis of samples containing CLA, and has the additional advantage of being a micro-preparative technique. Retention in Ag-HPLC is due primarily to the formation of transient complexes between the Ag^+ ions in the HPLC column and unsaturation (pi electrons) in the sample molecules. In general, the more unsaturation (double bonds), the more strongly the molecule is retained, though conjugated double bonds are an exception. Double bond configuration is



◀ **Fig. 2** Comparison of partial gas chromatograms of the CLA methyl ester region from cheese total fatty acid methyl esters with flame-ionization detection (*top*) and the same region with high-resolution selected-ion recording (*SIR*) at *m/z* 294.2559 (molecular weight of CLA methyl ester) (*bottom*). Separations were performed using a CP-Sil 88 capillary column (100 m × 0.25 mm id × 0.2 μm film thickness; Chrompack Inc., Raritan, NJ) using hydrogen as carrier gas. For gas chromatography, the oven temperature was held at 75°C for 2 min, programmed at 5°C/min to 180°C, held at 180°C for 33 min, programmed at 4 °C/min to 225 °C, and finally held at 225 °C for 43.8 min. For GC–MS, the oven temperature was held at 75 °C for 2 min, programmed at 5 °C/min to 170 °C, held at 170 for 40 min, programmed at 5 °C/min to 220 °C, and finally held at 220 °C for 20 min. Peaks indicated by an *asterisk* were not found in the SIR chromatogram and are not CLA isomers. CLA isomers in brackets were detected at low levels. Redrawn from Roach et al. [8] with permission of the authors and Lipids. Some identities have been annotated using information from Delmonte et al. [14] and Sehat et al. [11]

Table 1 Relative retention times (RRT) of CLA methyl ester isomers on a 100 m CP-Sil 88™ column

Isomer	RRT	Isomer	RRT
7*c*9*t*	1.083	9*c*11*c*	1.137
8*c*10*t*	1.085	10*c*12*c*	1.145
6*c*8*t*	1.086	13*c*15*t*	1.147
6*t*8*c*	1.086	12*t*14*c*	1.149
7*t*9*c*	1.091	11*c*13*c*	1.151
9*c*11*t*	1.091	12*c*14*c*	1.159
8*t*10*c*	1.097	13*t*15*c*	1.166
10*c*12*t*	1.104	13*c*15*c*	1.166
9*t*11*c*	1.109	12*t*14*t*	1.171
11*c*13*t*	1.114	11*t*13*t*	1.184
12*c*14*t*	1.118	13*t*15*t*	1.184
10*t*12*c*	1.122	9*t*11*t*	1.191
6*c*8*c*	1.128	8*t*10*t*	1.191
8*c*10*c*	1.131	10*t*12*t*	1.192
7*c*9*c*	1.131	6*t*8*t*	1.192
11*t*13*c*	1.136	7*t*9*t*	1.193

RRT relative to γ-linolenic acid methyl ester (GLA) calculated using $(RT_{isomer}-RT_{solvent})/(RT_{GLA}-Rt_{solvent})$. Oven temperature programme: 75 °C for 2 min, then 5 °C/min to 175 °C, holding at 175 °C for 33 min, then at 5 °C/min to 225 °C and finally holding at 225 °C for 8 min. Adapted from Delmonte et al. [14]

also important, with *cis* double bonds retained more-strongly than *trans*. Ag-HPLC utilizing columns packed with 5–10 μm Nucleosil SA™ or a similar material, with phenylsulfonic acid groups bonded to a silica substrate, in which the sulfonic acid protons have been exchanged with Ag^+ ions, is an especially useful technique for the separation and isolation of *cis* and *trans* geometrical and positional isomers of CLA as FAMEs and, to a lesser extent, as free fatty acids (FFAs) and triacylglycerols (TAGs). The technique has been reviewed in relation to lipids in general [16, 17] and CLA specifically [18].

One of the most popular Ag-HPLC columns is the ChromSpher Lipids™ column (Cat. No. 28313; 4.6 mm ID × 250 mm stainless steel; 5 μm particle size), which can be purchased from Varian-Chrompack International, Middelburg, The Netherlands. Many analysts recommend

the employment of two such columns connected in series to improve sample capacity and to provide the required peak-to-peak resolutions, but up to six columns in series have been used for enhanced separations [12]. Typical isocratic flow rates of 1.0–2.0 ml/min and compositions of 0.1–1.0% acetonitrile in hexane in the mobile phase, maintained at a constant temperature of ~23 °C, are adjusted to maintain pump head pressures at ≤ 2,500 psi and to maintain total sample elution times of 35 min or less. Void volumes are approximately 1.0 ml per column.

In a solvent system of acetonitrile in hexane, the acetonitrile competes with the Ag^+ ions for the unsaturated sites of the sample molecules. The more acetonitrile in the solvent, the faster the sample elutes from the column. With dienoic fatty acids, the greater the distance between the double bonds (up to three methylene groups at least), the more strongly they are retained. Thus, fatty acids with conjugated double bonds elute before those with methylene-interrupted double bonds, and the expected elution order (*trans*,*trans*-, then *cis*,*trans*/*trans*,*cis*-, then *cis*,*cis*-) is observed. These arguments also apply to the three fatty acid molecules attached to a glycerol backbone of TAG (ABA, AAB, ABC, etc.), but the location is also important. Fatty acids on the 1(3)-positions of the TAG molecule (the "A" in ABA) shield that in the 2-position (the "B") from the Ag^+ ions. So, while oleic acid (O; 9*c*-18:1) is retained more strongly than stearic acid (S; 18:0), for TAGs composed of two O and one S fatty acids, the elution order is SOS before SSO.

Separation as Methyl Esters

Ag-HPLC analysis of CLA isomers as methyl esters (or other ester derivatives [11, 19, 20]) continues to be the single largest application for this technology, an excellent example of which is illustrated in Fig. 3. Utilizing three Ag-HPLC columns connected in series and an isocratic solvent system of 0.1% acetonitrile in hexane, Eulitz and co-workers [12] were able to separate the very difficult 9*c*,11*t*- and 9*t*,11*c*-18:2 isomer pair as FAMEs, and, by utilizing two elution systems, others were able to determine the relative retention orders of all 32 *cis*/*trans* 6,8- to 13,15-CLA isomers [21]. Two to six columns connected in series were required to separate such isomer pairs as 8*t*,10*c*- from 7*t*,9*c*- and 11*c*,13*t*- from 9*t*,13*c*-18:2. In comparison, analysis of this mixture by GC utilizing a 100 m, highly polar capillary column could separate the *cis*/*cis*, the *cis*/*trans* and *trans*/*cis* (incomplete separation) and *trans*/*trans* 11,13-18:2 isomers, but could not resolve *trans*/*trans* 8,10-, 9,11- or 10,12-18:2. Propionitrile or butyronitrile in heptane may give a more stable performance as mobile phases (although these solvents are more much toxic than acetonitrile) [22].

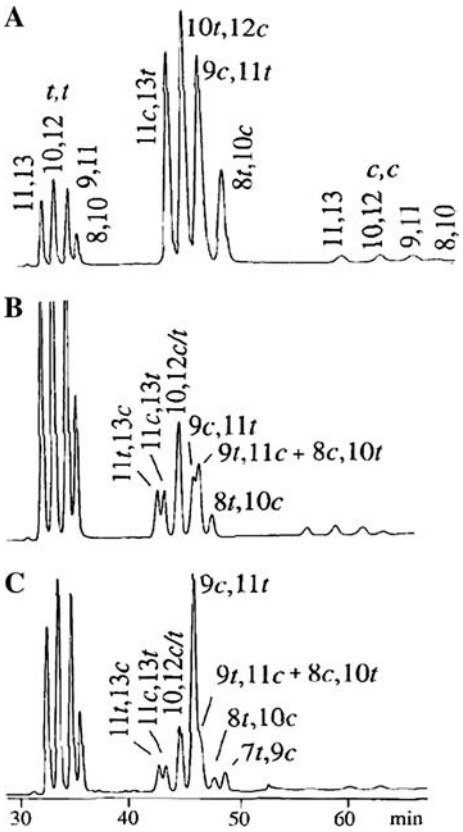


Fig. 3 Partial Ag-HPLC profile of (**A**) the methyl esters of a commercial CLA isomer mixture, (**B**) of the I_2-isomerized product of (**A** and **C**) of the I_2-isomerized product of (**A**) co-injected with cheese total lipid FAME. Three Chromspher Lipids™ columns in series with a mobile phase of 0.1% acetonitrile in hexane (1 ml/min) and detection by UV spectroscopy at 234 nm (with the permission of the authors and AOCS Press, and redrawn from the original) [12]

In the analysis of CLA in tissue samples by such methods, especially at low concentrations, it has been recommended that the results should be confirmed by GC and/or GC–MS to eliminate errors due to false positives [22].

Ag-HPLC (4.6 × 250 mm columns) may also be used for the semi-preparative isolation of CLA esters. Two Ag-HPLC columns connected in series were also used to isolate milligram quantities of CLA isomers from a mixture of 78.8% 9*t*,11*t*-18:2/21.2% 9*c*,11*t*-18:2 (both 17,17,18,18-d_4) (Fig. 4) [23]. Resolution of the two CLA isomers, which decreased with increasing weights of samples injected, was maintained at >95% of baseline by decreasing the percentage of acetonitrile in the isocratic hexane/acetonitrile solvent system. Purities of the isolated fractions were found to be >96% by GC analysis. A 10 mg sample was fractionated within 35 min using a solvent system of 1.0 ml/min of 0.15% acetonitrile in hexane. One could continue to compensate for losses in peak-to-peak resolution with increased sample sizes by

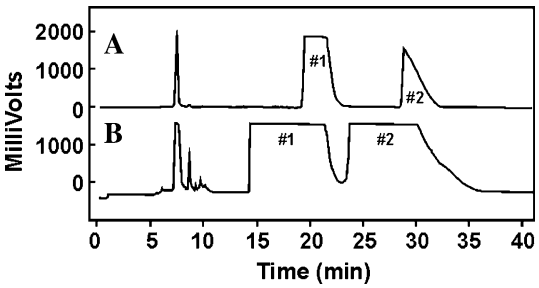


Fig. 4 Fractionation of a CLA sample by dual column Ag-HPLC. Sample sizes: 5 mg (**A**) and 10 mg (**B**). Flow rate: 1.0 ml/min 0.15% acetonitrile in hexane. UV detection at 215 and 212 nm, respectively. Peak #1 = 9-*trans*,11-*trans*-18:2; #2 = 9-*cis*,11-*trans*-18:2 (Published with the permission of Elsevier Ltd. and redrawn from the original) [23]

decreasing the percentage of acetonitrile in the solvent; but a 15 mg sample of the CLA isomer mixture (1.0 ml/min 0.1% acetonitrile in hexane) required some 80 min to elute. Two 10 mg samples could actually be fractionated within 70 min (2 × 35 min), less time than that required to separate one 15 mg sample.

Separation of Free Fatty Acids

More limited work has been done with the underivatized fatty acids. Using a single Chromspher Lipids™ Ag-HPLC column and isocratic solvent systems of hexane, acetonitrile (0.0–0.025%) and acetic acid (3.0–2.5%) and with UV detection at 234 nm, Cross and co-workers [24] fractionated a mixture of CLA isomers (8*t*,10*c*-, 9*c*,11*t*-, 10*t*,12*c*- and 11*c*-13*t*-18:2) in free fatty acid form. The resulting pattern was similar to that obtained with CLA FAME with acetonitrile in hexane as solvent (see Fig. 3). The FA composition of the isolated fractions, determined after conversion to FAME (BF_3/methanol) and analysis by GC, were also identical to that obtained for CLA as FAME. The same group [25] utilized Ag-HPLC and a similar solvent system to fractionate mixtures of CLA isomers obtained from commercial mixtures and from biological samples. The most abundant isomers, *cis/trans* 10,12-18:2 and *cis/trans* 9,11-18:2, were separated better as free acids on a single column than in the methyl ester form. However, a diminution in retention over time of the Ag-HPLC columns was noted, possibly as a result of a loss of silver ions because of the acetic acid in the mobile phase [25].

Separation of Triacylglycerols

By utilizing Ag-HPLC, successful analysis of seed oils high in conjugated dienoic or trienoic fatty acids has been achieved. Thus, Joh and co-workers [26] used a Nucleosil™ 5SA column (4.6 × 250 mm) saturated with silver ions and a complex gradient system of dichloromethane/dichloroethane/acetone/acetonitrile (1 ml/min) to separate TAGs from the Chinese melon [*Momordica charantia*; 57.1 mol% conjugated triene, primarily α-eleostearic (9*c*,11*t*,13*t*-18:3)]. Ag-HPLC (1.5 ml/min of 1.0% acetonitrile in hexane) was also utilized to fractionate a commercially available CLA-enriched TAG formulation (G-80; Loders Croklaan B.V., The Netherlands), as illustrated in Fig. 5 [27]. The elution patterns were achieved with either three (Fig. 5 Inset A) or four (Fig. 5 and Inset B) Ag-HPLC columns connected in series. A minimum of three columns in series were required to achieve ca. 50% separation (Fig. 5 Inset A) of peaks #3 and #4, but no fractionation of the major TAG peak (peak #4) was noted. Utilizing four Ag-HPLC columns connected in series resulted in a pattern of four distinct minor and one major peak (Fig. 5). The fractions were collected (the major peak was divided into three fractions, 2A1, 2A2 and 2A3), converted to FAMEs and analysed by GC. Peak #1 = mono-CLA/2 misc FAs, peak #2 = di-CLA/mono-saturated FA, peak #3 = di-CLA/9*c*-18:1, peak #4 = Tri-CLA, and peak #5 = di-CLA/mono *cis/cis* CLA isomer. (In this instance, the term "CLA" is used for both the 9,11- and the 10,12-isomers, and *cis/cis*

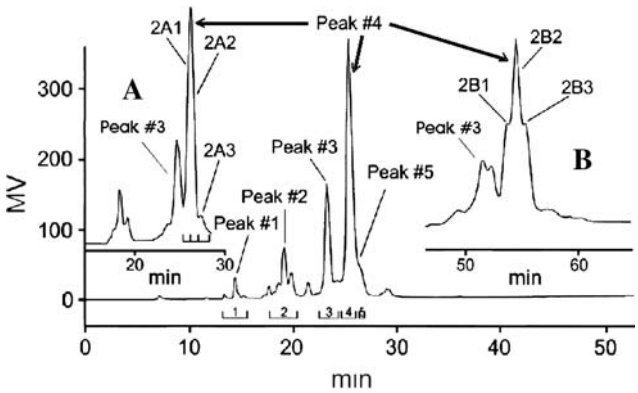


Fig. 5 Analysis of a commercial triacylglycerol formulation containing CLA by four-column Ag-HPLC [27]. Sample size: 100 µg. Flow rate 1.5 ml/min, 1.0% acetonitrile in hexane. UV detection at 206 nm. Peak #1 = mono-CLA with two miscellaneous fatty acids, peak #2 = di-CLA/mono-saturated fatty acid, peak #3 = di-CLA/mono 9c-18:1 and peak #4 = Tri-CLA; peak #5 = Tri-CLA. *Inset A* three-column Ag-HPLC. Sample size, 50 µg; flow rate 2.0 ml/min, 0.6% acetonitrile in hexane. Fraction 2A1 = 54/37%:10*t*,12*c*-18:2/ 9*c*,11*t*-18:2; fraction 2A2 = 36/56%:10*t*,12*c*-18:2/ 9*c*,11*t*-18:2; fraction 2A3 = 75/11%; di-9*c*,11*t*-18:2/mono-*cis/cis*-CLA (where *cis/cis* refers to 9*c*,11*c*- and 10*c*,12*c*-18:2). *Inset B* four-column Ag-HPLC. Sample size, 50 µg; flow rate 1.5 ml/min, 0.7% acetonitrile in hexane. Note: The two major peaks in *Insets A* and *B* are peaks #3 and #4 of the main chromatogram and illustrate changes in separation due to changes in the number of columns used or in solvent composition. (Published with permission of Elsevier Ltd, and redrawn from the original)

refers to 9*c*,11*c*- and 10*c*,12*c*-18:2). At 0.6% acetonitrile (1.5 ml/min), the desired peak could not be eluted within 120 min. At 0.7% acetonitrile (1.5 ml/min), the peak #4 eluted at 55 min and was partially fractionated into three peaks (Fig. 5 Inset B; <10% resolution; 1:2:1 ratio). Fractionation of the individual TAG isomers was improved, but at a cost of increased elution time. Solvent flow rates and compositions could be adjusted to yield total TAG elution patterns within 30–35 min and to maintain a system pressure < 2,500 psi. Comparable methods could no doubt be used for other lipid classes after conversion to suitable non-polar derivatives.

Concentration of Natural CLA Isomers for Further Analysis

In order to have sufficient material for analysis with tissue samples containing CLA at low levels, a pre-concentration step may be required. This can be accomplished either by subjecting the methyl esters to reversed-phase HPLC or Ag chromatography or at times by using the two techniques sequentially [28].

In reversed-phase chromatography, CLA isomers elute close to linoleate and can be collected preparatively by collecting a single broad C_{18} diene fraction. Many analysts add water to the mobile phase or use acetonitrile–water gradients. However, acetonitrile alone is preferable either at a constant flow rate or with a flow gradient, as there is no sacrifice of resolution and it is easier to recover the required esters by evaporation of the mobile phase. Most columns of the octadecylsilyl (ODS) type can be used for methyl esters of fatty acids [28], and base-stabilized ODS columns are of value for use with DMOX derivatives and picolinyl esters (but not free fatty acids) [29]. Evaporative light-scattering detection with a stream splitter can be used, or UV detection at 206 nm (isolated double bonds) or 230 nm (conjugated double bonds specifically), to collect appropriate fractions. Keeping the column at a constant temperature aids the reproducibility of the separation but is not essential for adequate resolution. On a standard analytical column (4.6 mm diameter), about 1 mg of sample can be separated in micro-preparative mode, but up to 20 mg can be chromatographed on a preparative column (10 mm diameter), as illustrated in Fig. 6 [5].

Ag thin-layer chromatography (TLC) and HPLC methods are available to obtain a concentrate of CLA as well as for analysis (see below), but they are needlessly complex for many purposes. A simple small-scale solid-phase extraction method adapted from a published procedure can be recommended in which an adsorbent with bonded phenylsulfonate groups is utilized in the silver ion form [30]. This can be applied to any methyl ester preparation, and to the C_{18} diene fraction isolated by reversed-phase HPLC as above, as the CLA isomers tend to elute with the monoene fraction rather than with the methylene-interrupted dienes in this instance.

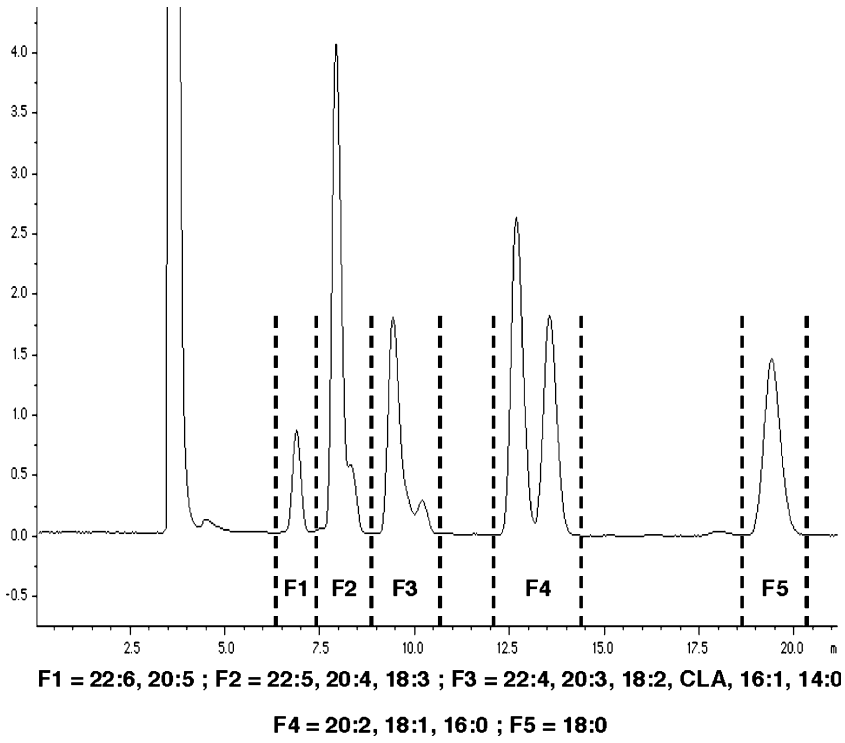


Fig. 6 Reversed-phase HPLC separation of fatty acid methyl esters including CLA. A Nucleosil C_{18} column (250 × 10 mm ID; 5 μm particles) was used with acetonitrile as mobile phase, and UV detection at 234 nm. Methyl esters (20 mg) in acetone were injected, with acetonitrile as mobile phase and a flow rate of 4 ml/min. The fraction corresponding to the C_{18} dienes may also contain some 14:0, 16:1 and certain polyunsaturated fatty acids [28]

Reversed-Phase HPLC With Second Derivative UV Detection

Conjugated dienes exhibit a distinct UV absorbance in the region of 230–235 nm, while isolated double bonds absorb at 206–210 nm. As the latter tend to be present in most tissues at relatively high levels, they can still interfere with the analysis of CLA isomers in tissues. Murru et al. [31] solved the problem by taking the differential of the first derivative spectrum and calculating a second derivative with two distinct peaks with minima at 234 and 242 nm. As the Beer–Lambert law is unaffected by differentiation, this technique enabled sensitive and accurate estimation of the conjugated diene content of fatty acids. In combination with reversed-phase HPLC, this is a powerful technique both for isolation and estimation of CLA and especially of CLA metabolites formed by elongation and desaturation of CLA in animal tissues, e.g., 9*c*,11*t*-18:2, 6*c*,9*c*,11*t*-18:3, 8*c*,11*c*,13*t*-20:3, and 5*c*,8*c*,11*c*,13*t*-20:4, together with the corresponding fatty acids formed by chain elongation and desaturation of 10*t*,12*c*-18:2. The 18:2 and 20:3 isomers tend to elute together, as do 18:3 and 20:4, but subsequent separation of these by GC is straightforward.

Gas Chromatography–Mass Spectrometry

Although GC with appropriate standards will allow some tentative identification of CLA isomers to be made, GC–MS is essential for structural confirmation and for detecting overlapping isomers. The EI mass spectra of the methyl esters of CLA isomers are qualitatively indistinguishable from one another and from methyl linoleate. Although there may be some differences in the relative intensities of some ions, these are of limited value in the analysis of CLA mixtures. However, they are useful for distinguishing CLA peaks from other fatty acids that elute in the same region of the chromatogram. In order to distinguish between different CLA positional isomers, specific derivatives of either the carboxyl group or the conjugated diene system have been formed. The most useful derivatives of the carboxyl group for determining the structure of unsaturated fatty acids contain nitrogen atoms [29]. Following ionization, radical-site-induced cleavage occurs at every carbon in the chain, and by applying simple rules to the resultant mass spectrum, the structures of unknown fatty acids can usually be deduced from first principles without having to refer to the mass spectra of standard compounds. DMOX derivatives afford the most informative mass spectra for distinguishing CLA isomers and have the added advantage that the chromatographic resolution is comparable to methyl esters at only slightly higher column temperatures [29].

DMOX derivatives are easily prepared by reacting the free fatty acids or esters/intact lipids with an excess of 2-amino-2-methyl-1-propanol at 190°C for 6 and 18 h, respectively, followed by addition of water, extraction of the derivatives with diethyl ether/isohexane (1:1) and washing the extract with water [5]. The products should be dried and stored over anhydrous sodium sulfate to prevent ring opening. Although no adverse effects of using high temperatures for derivatizing CLA have been reported, other milder preparation procedures are available [32].

The mass spectra of DMOX derivatives of CLA contain an intense ion at m/z = 113 corresponding to a McLafferty rearrangement ion and a molecular ion at m/z = 333 (confirming a diunsaturated C_{18} structure) [33]. There is a series of ions between m/z = 318 ($[M-15]^+$ ion) and m/z = 126, each corresponding to chain fragments with one less carbon. In a saturated part of the chain, the difference between adjacent ions is 14 amu, corresponding to loss of a methylene group, but the pattern is altered when a double bond is encountered. If a double bond occurs between carbons at positions n and $n + 1$ carbons (counting from the carboxyl end of the molecule) then there is a gap of 12 amu between ions containing n-1 and n carbons. Thus in 10,12-18:2 the double bonds at Δ10 and Δ12 give rise to gaps of 12 amu between m/z values corresponding to C-9 (m/z = 210) and C-10 (m/z = 222), and C-11 (m/z = 236) and C-12 (m/z = 248) fragments, respectively (Fig. 7). Another characteristic feature is the presence of two intense ions (at m/z = 276 and 290 for 10,12-18:2) containing $m + 2$ (where m denotes the first carbon of the distal double bond, i.e., due to cleavage allylic to the distal double bond) and $m + 3$ carbons. The allylic ion containing n-2 carbons (m/z = 196) (where n denotes the first carbon of the proximal double bond) is also often abundant. The same patterns hold for the published mass spectra of the DMOX derivatives of isomers from 6,8-18:2 to 13,15-18:2 except that the $m + 2$ ion predominates over the $m + 3$ ion as the diene system moves towards the extremities of the molecule, i.e., for 6,8-18:2 to 8,10-18:2 and 13,15-18:2.

Conjugated dienes react with α,β-unsaturated carbonyl compounds to form six-membered ring adducts by a cycloaddition reaction known as the Diels–Alder reaction. CLA (as the methyl esters) readily react with the dienophile, MTAD thus fixing the position of the double bond, and the resultant adducts give informative mass spectra [34, 35]. The reaction is carried out by simply briefly mixing CLA with a solution of MTAD in dichloromethane at 0°C and stopping the reaction with 1,3-hexadiene. The adducts are analysed on a non-polar column (30 m DB5-MS™) at high temperatures (160 °C for 3 min then programmed at 5 °C/min to 350 °C). There is a strong molecular ion at m/z = 407 and an $[M–CH_3O]^+$ ion at m/z = 376. Cleavages alpha to the ring give rise to two intense $[M-R_1]^+$ and

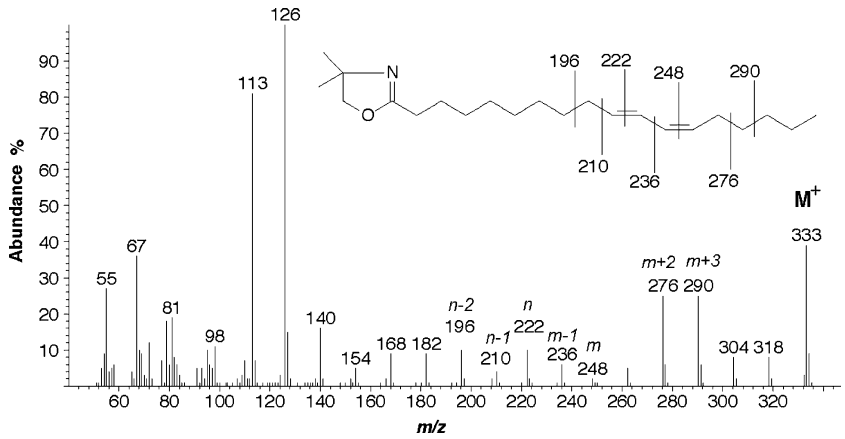


Fig. 7 Gas chromatography-electron-impact mass spectrum of the 4,4-dimethyloxazoline (*DMOX*) derivative of *trans*-10, *cis*-12-18:2 from a commercial CLA mixture

$[M\text{-}R_2]^+$ ions (where R_1 corresponds to the alkyl moiety and R_2 the methyl ester part of the chain), readily allowing the original position of the diene system to be deduced. Loss of methanol from $[M\text{-}R_1]^+$ produces another prominent ion ($[M\text{-}R_1\text{–}CH_3OH]^+$). Thus, for 9,11-18:2 and 10,12-18:2 the $[M\text{-}R_1]^+$, $[M\text{-}R_2]^+$ and $[M\text{-}R_1\text{–}CH_3OH]^+$ ions occur at *m/z* = 322, 250 and 290 and *m/z* = 336, 236 and 304, respectively (Fig. 8).

MTAD adducts are especially useful for commercial CLA preparations, particularly for determining 8,10-18:2 which is difficult to separate from 9,11-18:2 by GC as methyl ester or DMOX derivatives [34]. Adducts of isomer mixtures occur as a single chromatographic peak (there is a small degree of separation within the peak) and individual isomers are identified by using reconstructed ion chromatograms (RICs) of the $[M\text{-}R_1]^+$ and $[M\text{-}R_2]^+$ ions. It is possible to estimate the different positional isomers semi-quantitatively using the summation of the areas of the $[M\text{-}R_1]^+$ and $[M\text{-}R_2]^+$ ions, although because of the lack of chromatographic resolution it can be difficult to be certain about the presence of isomers at very low levels. For isomers ranging from 7,9- to 11,13-18:2 good agreement with ^{13}C NMR spectroscopy was obtained for a highly isomerized CLA mixture, although NMR had the advantage that geometrical isomers could be distinguished [36]. In the GC–MS approach, geometrical isomers are not distinguished, the ions representing a particular positional isomer being derived from the sum of the geometrical isomers. However, it may be possible to utilize the claim that *cis*,*trans*/*trans*,*cis* and *trans*,*trans* isomers give MTAD adducts of different stereochemistry (*trans* and *cis* isomers of the chains about the ring, respectively) that can be resolved by GC–MS [35].

Tissue or food samples containing CLA at natural or slightly elevated levels do not appear to have been analysed as the MTAD adducts by GC–MS. Such an approach is likely to be problematic, partly because of the relatively low abundance of isomers compared to the major 9*c*11*t*-18:2 isomer and partly because it has been observed that MTAD may react with methylene-interrupted unsaturated fatty acids if conditions are too harsh [34].

Because CLA isomers in both commercial and natural CLA invariably overlap by GC there has been use of reconstructed ion chromatograms of specific ions to aid identification of CLA isomers in the form of DMOX derivatives. Roach and colleagues [37] have highlighted

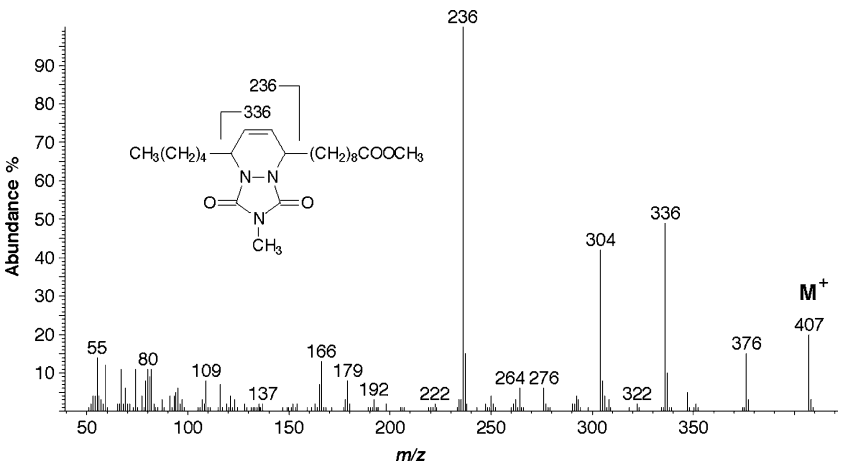


Fig. 8 Gas chromatography-electron-impact mass spectrum of the 4-methyl-1,2,4-triazoline-3,5-dione (*MTAD*) adduct of 10,12-18:2 methyl ester from a commercial CLA mixture

seven diagnostic ions per isomer, giving a total of 28 ions (56 ions were highlighted but some occurred more than once) for isomers ranging from 6,8- to 13,15-18:2, which are useful for identifying positional isomers (Table 2). The two pairs of ions (containing $n-1$ and n and $m-1$ and m carbons) each differing by 12 amu due to fragmentation around the double bonds can be used, but the allylic cleavage ion containing $m + 2$ carbons and that with $m + 3$ carbons are often more useful, especially for detecting isomers at trace levels, because of their greater intensity. However, the m/z values for the latter ions are not specific to any one positional isomer and therefore, some degree of chromatographic separation, which is often the case, is useful. Indeed, after locating an isomer using the latter ions, signal contributions from adjacent peaks can be subtracted so that a representative mass spectrum of the isomer can be obtained to confirm its identity. As an example, the RIC for the allylic cleavage ion ($m/z = 234$) clearly showed the presence of two 7,9-18:2 *cis*,*trans*/*trans*,*cis* isomers at the leading edge of the major 9*c*,11*t*-18:2 peak (allylic ion $m/z = 262$) in a variety of materials [38]. The 7*t*,9*c*-18:2 isomer, found to be the second major isomer at about 7% of the total CLA, had previously gone unnoticed because it was masked by the major 9*c*,11*t*-18:2 peak. In addition, a rapid, specific and sensitive high-resolution GC–MS method using selected ion recording (SIR) of the molecular ion of CLA methyl ester (m/z 294.2559) was developed to unambiguously differentiate CLA isomers from non-CLA peaks in total cheese FAMEs [8] (Fig. 2).

DMOX derivatives, picolinyl esters and MTAD adducts have all been used, often in a complementary manner, to analyse the metabolites formed by elongation and desaturation of CLA following suitable fractionation by HPLC [4]. For example, the metabolites isolated from rats fed a commercial CLA containing a mixture of isomers were examined as the DMOX derivatives. The structures of 8,11,13-20:3 (derived from 9,11-18:2), and 8,12,14-20:3 and 5,8,12,14-20:4 (derived from 10,12-18:2) acids were readily determined by using the "12 amu rule". For example, the molecular ion for the DMOX derivative of 8,12,14-20:3 was at $m/z = 359$ and gaps of 12 amu between m/z 182 and 194, 236 and 248, and 262 and 274 located the double bonds at C-8, C-12 and C-14, respectively. The base peak at $m/z = 222$ was due to cleavage between C-10 and C-11, i.e., at the centre of the *bis*-methylene-interrupted double bond system. However, the complete structure of a 5,8,11,13-20:4 acid (derived from 9,11-18:2) could not be elucidated from the mass spectrum of the DMOX derivative. The positions of all double bonds were determined from the mass spectrum of the picolinyl ester only after deuteration of the double bonds, gaps of 15 amu locating the positions of methylene groups containing a deuterium atom. Additionally, the position of the conjugated system was confirmed from the mass spectrum of the MTAD adduct of the methyl ester. The molecular ion was at $m/z = 431$ and the $[M\text{-}R_2]^+$ ion at $m/z = 250$ corresponded to a hexyl chain attached to the ring, thus confirming an 11,13 conjugated double bond system. The intensity of the $[M\text{-}R_1]^+$ ion ($m/z = 346$) was particularly weak.

A powerful tandem mass spectrometry (MS/MS) technique using acetonitrile chemical ionization was developed for determining double bond position and geometry in CLA isomers from natural samples [39]. The acetonitrile generated a (1-methyleneimino)-1-ethenylium ion which reacted with the CLA isomers, as the methyl esters, to form a $[M + 54]^+$ ion which upon collisionally activated dissociation produced two ions as a result of carbon–carbon cleavage at positions vinylic to either side of the conjugated diene system. The two ions were characteristic of the

Table 2 Diagnostic ions for DMOX derivatives of CLA positional isomers ranging from 6,8-18:2 to 13,15-18:2

Isomer	$n-2$ ion (m/z)	$n-1$ ion (m/z)	n ion (m/z)	$m-1$ ion (m/z)	m ion (m/z)	$m + 2$ ion (m/z)	$m + 3$ ion (m/z)
6, 8[a]	140	154	166	180	192	220	234
7, 9[b]	154	168	180	194	206	234	248
8, 10[c]	168	182	194	208	220	248	262
9, 11	182	196	208	222	234	262	276
10, 12	196	210	222	236	248	276	290
11, 13	210	224	236	250	262	290	304
12, 14	224	238	250	264	276	304	318
13, 15	238	252	264	278	290	318	332

The molecular ion is $m/z = 333$

Adapted from Roach et al. [37]

[a] m/z 154 is weak and $m/z = 220$ is more abundant than $m/z = 234$

[b] m/z 154 is weak and $m/z = 234$ is more abundant than $m/z = 248$

[c] m/z 248 is more abundant than $m/z = 262$

position of the double bonds, one (α) containing the ester group, the other (ω) the terminal methyl group. The ion resulting from vinylic cleavage to a *trans* double bond was more abundant than that from a *cis* double bond. Therefore, the ratio of the abundance of α to that of ω was highest (>4.8) for a *cis*,*trans* arrangement, lowest (<0.5) for *trans*,*cis* and intermediate (0.7–3.2) for *cis*,*cis* and *trans*,*trans*, thus allowing differentiation of the geometrical isomers (with pure standards at least). When used in conjunction with GC retention time data the majority of CLA isomers in milk samples were identified

Distinguishing *cis* and *trans* Double Bonds

While infrared spectroscopy methods are especially useful for confirming the presence of *trans* double bonds in fatty acids, they do not help to locate the specific position in the fatty acid chain. For this purpose, there is no alternative to using chemical degradative techniques. The first step is to obtain a pure single component either by reversed-phase or silver ion chromatography (or by a combination of both). The CLA isomer must then be subjected to partial hydrogenation with hydrazine to yield a mixture of *cis* and *trans* monoenes, which can be easily separated by silver ion chromatography and identified either by chemical oxidative procedures or by GC–MS. Procedures of this kind have been used to identify natural CLA isomers [40] and for the elongation products of CLA [4]. Mass spectrometry with acetonitrile-covalent adduct chemical ionization can also be helpful though not necessarily definitive [39].

Nuclear Magnetic Resonance Spectroscopy

^{13}C-NMR spectroscopy has proven to be the single most comprehensive method for the identification and quantification of all the positional (7,9- to 11,13-18:2) and geometrical isomers (*cis*,*trans*-, *trans*,*cis*-, *cis*,*cis*- and *trans*,*trans*-) present in commercial CLA preparations [36]. This is arguably the most comprehensive analytical procedure for CLA, but unfortunately the methodology requires substantial amounts of sample and is not likely to be applicable to tissue extracts at natural levels.

Acknowledgments This work has been funded in part by the Scottish Executive Environment and Rural Affairs Department.

References

1. Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW, Nelson GJ (eds) (1999) Advances in conjugated linoleic acid research, vol 1. AOCS Press, Champaign
2. Sebedio JL, Christie WW, Adlof RO (eds) (2003) Advances in conjugated linoleic acid research. vol 2, AOCS Press, Champaign
3. Yurawecz MP, Kramer JKG, Gudmundsen O, Pariza MW, Banni S (eds) (2006) Advances in conjugated linoleic acid research, vol 3. AOCS Press, Champaign
4. Sébédio JL, Juanéda P, Dobson G, Ramilison I, Martin JC, Chardigny JM, Christie WW (1997) Metabolites of conjugated isomers of linoleic acid (CLA) in the rat. Biochim Biophys Acta 1345:5–10
5. Christie WW, Sébédio JL, Juanéda P (2001) A practical guide to the analysis of conjugated linoleic acid (CLA). INFORM 12, 147–152. Available online at http://www.lipidlibrary.co.uk/
6. Shantha NC, Decker EA, Hennig B (1993) Comparison of methylation methods for the quantitation of conjugated linoleic acid isomers. J Assoc Off Anal Chem 76:644–649
7. Christie WW (1993) Preparation of ester derivatives of fatty acids for chromatographic analysis. In: Christie WW (ed) Advances in lipid methodology two. Oily Press, Dundee, pp 69–111
8. Roach JAG, Yurawecz MP, Kramer JKG, Mossoba MM, Eulitz K, Ku Y (2000) Gas chromatography–high resolution selected-ion mass spectrometric identification of trace 21:0 and 20:2 fatty acids eluting with conjugated linoleic acid isomers. Lipids 35:797–802
9. Yurawecz MP, Sehat N, Mossoba MM, Roach JAG, Kramer JKG, Ku Y (1999) Variations in isomer distribution in commercially available conjugated linoleic acid. Fett/Lipid 101:277–282
10. Lavillonniere F, Martin JC, Bougnoux P, Sébédio J-L (1998) Analysis of conjugated linoleic acid isomers and content in French cheeses. J Am Oil Chem Soc 75:343–352
11. Sehat N, Kramer JKG, Mossoba MM, Yurawecz MP, Roach JAG, Eulitz K, Morehouse KM, Ku Y (1998) Identification of conjugated linoleic acid isomers in cheese by gas chromatography, silver ion high performance liquid chromatography and mass spectral reconstructed ion profiles. Comparison of chromatographic elution sequences. Lipids 33:963–971
12. Eulitz K, Yurawecz MP, Sehat N, Fritsche J, Roach JAG, Mossoba MM, Kramer JKG, Adlof RO, Ku Y (1999) Preparation, separation and confirmation of the eight geometrical *cis/trans* conjugated linoleic acid isomers 8,10- through 11,13-18:2, Lipids 34:873–877
13. Destaillats F, Angers P (2003) Directed sequential synthesis of conjugated linoleic acid isomers from $\Delta^{7,9}$ and $\Delta^{12,14}$. Eur J Lipid Sci Technol 105:3–8
14. Delmonte P, Roach JAG, Mossoba MM, Losi G, Yurawecz MP (2004) Synthesis, isolation, and GC analysis of all the 6,8- to 13,15-*cis/trans* conjugated linoleic acid isomers. Lipids 39:185–191
15. Cruz-Hernandez C, Deng Z, Zhou J, Hill AR, Yurawecz MP, Delmonte P, Mossoba MM, Dugan MER, Kramer JKG (2004) Methods for the analysis of conjugated linoleic acids and *trans*-18:1 isomers in dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography. J AOAC Int 87:545–561
16. Nikolova-Damyanova B (1992) Silver ion chromatography and lipids. In: Christie WW (ed) Advances in lipid methodology-one. Oily Press, Dundee, pp 181–237
17. Nikolova-Damyanova B (2003) Lipid analysis by silver ion chromatography. In: Adlof RO (ed) Advances in lipid methodology-five. Oily Press, Bridgwater, pp 43–123
18. Adlof RO (2003) Application of silver ion chromatography to the separation of conjugated linoleic acid isomers. In: Christie W, Sébédio J, Adlof RO (eds) Advances in conjugated linoleic acid research, vol 2. AOCS Press, Champaign, pp 37–55

19. Yurawecz MP, Morehouse KM (2001) Silver-ion HPLC of conjugated linoleic acid isomers. Eur J Lipid Sci Technol 103:609–613
20. Nikolova-Damyanova B, Momchilova S, Christie WW (2000) Silver ion high-performance liquid chromatographic separation of conjugated linoleic acid isomers, and other fatty acids, after conversion to *p*-methoxyphenacyl derivatives. J High Resolut Chromatogr 23:348–352
21. Delmonte P, Kataoka A, Corl BA, Bauman DE, Yurawecz MP (2005) Relative retention order of all isomers of *cis/trans* conjugated linoleic acid FAME from the 6,8- to 13,15-positions using silver ion HPLC with two elution systems. Lipids 40:509–514
22. Müller A, Düsterloh K, Ringseis R, Eder K, Steinhart H (2006) Development of an alternate eluent system for Ag^+-HPLC analysis of conjugated linoleic acid isomers. J Sep Sci 29:358–365
23. Adlof RO (2004) Separation of conjugated linoleic acid methyl esters by silver-ion high performance liquid chromatography in semi-preparative mode. J Chromatogr A 1033:369–371
24. Cross RF, Ostrowska E, Muralitharan M, Dunshea FR (2000) Mixed mode retention and the use of competing acid for the Ag^+-HPLC analysis of underivatized conjugated linoleic acids. J High Resolut Chromatogr 23:317–323
25. Ostrowska E, Dunshea FR, Muralitharan M, Cross RF (2000) Comparison of silver-ion high-performance liquid chromatographic quantification of free and methylated conjugated linoleic acids. Lipids 35:1147–1153
26. Joh Y-G, Kim S-J (1998) Analysis of molecular species of triacylglycerols from vegetable oils containing fatty acids with non-methylene-interrupted double bonds, by HPLC in the silver-ion mode. J Jpn Oil Chem Soc 47:927–936
27. Adlof RO, Menzel A, Dorovska-Taran V (2002) Analysis of CLA-enriched triacylglycerol mixtures by silver-ion HPLC. J Chromatogr A 953:293–297
28. Juanéda P, Sébédio JL (1999) Combined silver-ion and reversed-phase high-performance liquid chromatography for the separation and identification of C_{20} metabolites of conjugated linoleic acid isomers in rat liver lipids. J Chromatogr B 724:213–219
29. Christie WW (1998) Gas chromatography–mass spectrometry methods for structural analysis of fatty acids. Lipids 33:343–353
30. Christie WW (1989) Silver ion chromatography using solid-phase extraction columns packed with a bonded-sulfonic acid phase. J Lipid Res 30:1471–1473
31. Murru E, Angioni E, Carta G, Melis MP, Spada S, Banni S (2003) Reversed-phase HPLC analysis of conjugated linoleic acid and its metabolites. In: Sébédio JL, Christie WW, Adlof RO (eds) Advances in conjugated linoleic acid research, vol 2. AOCS Press, Champaign, pp 94–100
32. Kuklev DV, Smith WL (2003) A procedure for preparing oxazolines of highly unsaturated fatty acids to determine double bond positions by mass spectrometry. J Lipid Res 44:1060–1066
33. Spitzer V, Marx F, Pfeilsticker K (1994) Electron impact mass spectra of the oxazoline derivatives of some conjugated diene and triene C_{18} fatty acids. J Am Oil Chem Soc 71:873–876
34. Dobson G (1998) Identification of conjugated fatty acids by GC–MS of 4-methyl-1,2,4-triazoline-3,5-dione adducts. J Am Oil Chem Soc 75:137–142
35. Reaney MJT, Liu Y-D, Taylor WG (2001) Gas chromatographic analysis of Diels–Alder adducts of geometrical and positional isomers of conjugated linoleic acid. J Am Oil Chem Soc 78:1083–1086
36. Davis AL, McNeill GP, Caswell DC (1999) Analysis of conjugated linoleic acid isomers by ^{13}C NMR spectroscopy. Chem Phys Lipids 97:155–165
37. Roach JAG, Mossoba MM, Yurawecz MP, Kramer JKG (2002) Chromatographic separation and identification of conjugated linoleic acid isomers. Anal Chim Acta 465:207–222
38. Yurawecz MP, Roach JAG, Sehat N, Mossoba MM, Kramer JKG, Fritsche J, Steinhart H, Ku Y (1998) A new conjugated linoleic acid isomer, 7 *trans*, 9 *cis*-octadecadienoic acid, in cow milk, cheese, beef and human milk and adipose tissue. Lipids 33:803–809
39. Michaud AL, Yurawecz MP, Delmonte P, Corl BA, Bauman DE, Brenna JT (2003) Identification and characterization of conjugated fatty acid methyl esters of mixed double bond geometry by acetonitrile chemical ionization tandem mass spectrometry. Anal Chem 75:4925–4930
40. Christie WW (1973) The structure of bile phosphatidylcholines. Biochim Biophys Acta 316:204–211